

Eudesmin attenuates *Helicobacter pylori*-induced epithelial autophagy and apoptosis and leads to eradication of *H. pylori* infection

JAI-SING YANG¹, CHAO-MIN WANG², CHIU-HSIAN SU³, HAN-CHEN HO⁴,
CHIUNG-HUNG CHANG^{5,6}, CHANG-HUNG CHOU^{2,3} and YUAN-MAN HSU³

¹Department of Medical Research, China Medical University Hospital; ²Research Center for Biodiversity; ³Department of Biological Science and Technology, China Medical University, Taichung 40402; ⁴Department of Anatomy, Tzu-Chi University, Hualien 97004; ⁵Department of Traditional Chinese Medicine, Taichung Veterans General Hospital, Taichung 40705; ⁶Department of Traditional Chinese Medicine, Tainan Municipal Hospital, Tainan 70173, Taiwan, R.O.C.

Received April 2, 2016; Accepted July 1, 2016

DOI: 10.3892/etm.2018.5701

Abstract. Eudesmin has been proven to possess anti-inflammatory effects. In the present study, the effects of eudesmin on *Helicobacter pylori* (*H. pylori*)-mediated autophagy, apoptosis, immune response and inflammation were determined in human gastric adenocarcinoma (AGS) cells *in vitro* and in C57BL/6 mice *in vivo*. Detection of the production of interleukin (IL)-8, IL-1 β and immunoglobulin M (IgM) was performed using ELISA. Identification of the activation of apoptosis-associated caspase-3, -8 and -9 proteins, Bcl-2-associated X protein (Bax) and BH3 interacting domain death agonist (Bid) protein, was determined through western blot analysis. Autophagy microtubule-associated protein 1A/1B-light chain 3, isoform B (LC-3B) expression was measured using immunostaining. The results of the present study demonstrated that eudesmin inhibited the growth of *H. pylori*, with increased inhibition activity against antibiotic resistant strains compared with the reference strain. In addition, *H. pylori*-induced IL-8 secretion, LC-3B expression and apoptosis-associated protein (caspase-3, -8 and -9, Bax and Bid) activation in AGS cells was suppressed by eudesmin. Furthermore, eudesmin suppressed IL-1 β and IgM production in *H. pylori*-infected C57BL/6 mice *in vivo*. In conclusion, eudesmin may be developed as a promising

therapeutic agent to prevent and/or treat *H. pylori*-associated gastric inflammation.

Introduction

Helicobacter pylori (*H. pylori*) infection is one of the most prevalent bacterial infections worldwide, and is a primary cause of gastritis, gastroduodenal ulcers and malignancies (1,2). *H. pylori* adhere to the gastric mucosa, inducing the production of reactive oxygen species (ROS) which damage the epithelium (3). The response of the gastric mucosal epithelium to *H. pylori* infection is a multistep progression reflecting the interaction of several factors, including bacterial virulence, specific receptor-linked signaling pathways and the host immune response (4,5). The first-line therapy for *H. pylori* infection is antibiotics, but the increasing emergence of antibiotic-resistant *H. pylori* strains has led to a decline in eradication rates (6,7). Therefore, developing alternative treatments for *H. pylori* infection is important.

Previous studies of novel *H. pylori* treatments have decreased *H. pylori*-triggered ROS production and apoptosis but enhanced autophagy (8,9). Apoptosis and autophagy are recognized to be non-inflammatory programmed cell-death (PCD) pathways (10,11). Apoptosis and autophagy serve vital roles in tissue homeostasis and in disease development in infected patients (9,10). Apoptosis (type I PCD) includes the cell-surface death receptor pathway and the mitochondrial pathway. The Fas/CD95 receptor, Fas ligand and downstream caspase-8 initiate the process of apoptosis in the death receptor-dependent pathway. The mitochondrial pathway is characterized by an increase in mitochondrial membrane permeability and the release of cytochrome *c* into the cytoplasm. Cytochrome *c* then initiates the formation of the apoptosome, which activates caspase-9. Finally, caspase-8 or -9 activate caspase-3, thus triggering apoptosis (8-10). Autophagy is a catabolic process encompassing the pathways for intracellular macromolecule degradation (9,12). Autophagy begins with the sequestration of cytoplasmic organelles in a membrane vacuole, forming an autophagosome, which then fuses with a lysosome, where

Correspondence to: Dr Yuan-Man Hsu, Department of Biological Science and Technology, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C.
E-mail: yuanmh@mail.cmu.edu.tw

Professor Chang-Hung Chou, Research Center for Biodiversity, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C.
E-mail: choumasa@mail.cmu.edu.tw

Key words: eudesmin, *Helicobacter pylori*, autophagy, apoptosis, inflammation

the cellular materials are degraded and recycled. Increased autophagic activity is associated with cell death, and autophagy is now considered type II PCD (9,12).

Eudesmin (Fig. 1) has previously been demonstrated to exert weak toxicity in mice and no toxicity in human macrophages (13). In immunological studies, eudesmin inhibits tumor necrosis factor (TNF)- α production and T cell proliferation (14). A previous study reported that eudesmin-induced vascular relaxation of rat aorta could be facilitated by the endothelial histamine receptor-mediated release of nitric oxide and prostanoids (15). (+)-Eudesmin can induce neurite outgrowth from PC12 cell neurons by stimulating signaling upstream of the mitogen-activated protein kinase, protein kinase C and protein kinase A pathways (16). However, there are no applicable studies on eudesmin regarding the response of epithelial cells to *H. pylori* infection. In the present study, the effects of eudesmin, extracted from *Fatsia polycarpa* Hayata, on *H. pylori*-induced epithelial damage, as well as *H. pylori* colonization *in vitro* [human gastric adenocarcinoma (AGS) cells] and *in vivo* (C57BL/6 mice) was investigated.

Materials and methods

Isolation and identification of eudesmin. The leaves of *Fatsia polycarpa* Hayata were collected in November 2009 from study sites in Hehuan mountain (2105 m above sea level), Hehuanshan, Taiwan. Air-dried leaves of *F. polycarpa* Hayata (7 kg total) were extracted with methanol over three times, following standard extraction procedures (17). The isolated compound was identified by ^1H NMR and ^{13}C NMR spectroscopy using a Varian Inova 600 (Bruker Daltonics Inc., Billerica, MA, USA) and electrospray ionisation mass spectrometry using a Bruker Daltonics Esquire HCT (Bruker Daltonics Inc.) as (+)-eudesmin, through comparison of spectra data in previously reported literature (18). Eudesmin was dissolved in 0.1% of dimethyl sulfoxide (DMSO) for cell culture experiments.

Bacterial strains, human cell lines and culture conditions. The *H. pylori* reference strain 26695 (ATCC 700392) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The antibiotic-resistant (metronidazole and clarithromycin) *H. pylori* strains V633, V1254, V1354 and V2356 were clinical isolates from previous studies (19,20). *H. pylori* were grown on Brucella agar (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 5% sheep blood under microaerophilic conditions at 37°C for 48-72 h. Brucella blood agar plates containing *H. pylori* supplement SR0147E (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) were used to examine the *H. pylori* load in infected mice under same culture condition. *Salmonella enterica* serovar Typhimurium (ATCC 6994), *Escherichia coli* (ATCC 25922) and *Streptococcus aureus* (ATCC 25923) were obtained from the ATCC and *Pseudomonas aeruginosa* (BCRC 13984) was obtained from the Bioresource Collection and Research Centre (Hsinchu, Taiwan). These bacteria were grown in Luria-Bertani medium (BD Biosciences) at 37°C for 48-72 h. Human AGS cells (CRL-1739; ATCC, Manassas, VA, USA) were purchased from ATCC and were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA),

supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Antimicrobial activity of eudesmin on Gram-negative and Gram-positive bacteria. The minimum inhibitory concentrations (MICs) of eudesmin were tested by a two-fold serial dilution method. Eudesmin was serially diluted with 0.1% DMSO to achieve concentrations of 500, 250, 125, 62.5, 31.25 and 15.625 μM . Equal volumes of bacterial suspension [1×10^6 colony forming units (CFUs)/ml] and diluted eudesmin samples were mixed and added to a 96-well plate, with an additional well containing broth only that acted as a negative control. The plate was incubated at 37°C for 24 h, following which the well containing the lowest concentration of eudesmin presenting with no visible bacterial growth was considered the MIC. The minimum bactericidal concentrations (MBCs) of eudesmin were then obtained. All samples with concentrations of eudesmin that exhibited complete inhibition of visual bacterial growth were identified and 50 μl of each culture was transferred onto a Mueller-Hinton agar plate supplemented with 5% sheep blood and incubated for 48-72 h at 37°C. The complete visual absence of bacterial colonies on the agar surface in the lowest eudesmin concentration was defined as the MBC. Each assay was repeated three times.

Scanning electron microscopy. *H. pylori* cultures were treated with 0 (control), 10, 20, 40, 80 and 250 μM eudesmin on Brucella blood agar plates under microaerophilic conditions at 37°C for 6 h. Bacterial colonies were scraped from the plates and then washed twice in phosphate-buffered saline (PBS) and bacteria were collected by centrifugation at 15,000 \times g for 10 min at room temperature. The pellets were then transferred to cover glasses and fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 h. Following rinsing with buffer, specimens were post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1.5 h at room temperature. Specimens were subsequently dehydrated with a graded series of ethanol up to 100% ethanol. Following two exchanges of 100% acetone, specimens were critical point dried and sputter coated with gold. Specimens were then observed under a scanning electron microscope (Hitachi S-4700; Hitachi, Ltd., Tokyo, Japan) at 15 kv. Different areas (≥ 3) were randomly selected for image capture at magnification of $\times 10,000$ and representative images were selected.

Cell viability assay. AGS cells were seeded into 96-well plates at a density of 1×10^4 cells/well and cultured in RPMI 1640 medium in an incubator containing 5% CO_2 at 37°C for 18 h. Eudesmin in 0.1% DMSO at concentrations of (5, 10, 20, 40, 80, 160, 320 and 640 μM) was then added to cells and then cultured at 37°C in CO_2 for 24 h. A control group, which was treated only with 0.1% DMSO, underwent the same procedures. To determine cell viability, the trypan blue exclusion test was used, where results represent the percentage of cells surviving treatment. Equal volumes of 10 μl cell suspension in PBS (pH 7.4) and trypan blue (Thermo Fisher Scientific, Inc.) were mixed. Subsequently, stained (dead) and unstained (surviving) cells were counted using a hemocytometer.

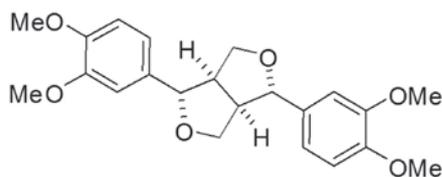


Figure 1. Structure of eudesmin.

Association activity assay. AGS cells and PBS-suspended *H. pylori* 26695 at a multiplicity of infection (MOI) ratio of 100 were co-cultured in antibiotic-free RPMI-1640 medium supplemented with 10% FBS. The 6 concentrations of eudesmin (0, 10, 20, 40, 80 and 250 μM) were added to the culture. A control group, which was treated only with 0.1% DMSO, underwent the same procedures. Cell-associated bacteria were quantified 6 h later, following infection of the host (AGS) cells by osmotic lysis. Cell culture supernatants were removed by centrifugation at 1,500 \times g for 5 min at room temperature, cells were washed with PBS twice and osmotic lysis was performed to calculate the total quantity of bacteria remaining. For this purpose, sterile water was added to the infected cells following washing, the cell lysates were re-suspended in PBS, and then plated using serial dilutions on the Brucella blood agar plates. These plates were cultured for 100 μl from each dilution at 37°C for 48 h. Bacterial cell numbers were then determined by manual colony counting. The association activity of *H. pylori* was determined as the mean of triplicate readings at each concentration of eudesmin. The bacteria associated with host cells included adherent and invading bacteria. The results are expressed as a percentage of the association activity of *H. pylori* in comparison with the control group.

Confocal fluorescence microscopy. AGS cells were grown on glass coverslips ($\sim 5 \times 10^6$ cells/dish) for 18 h at 37°C. *H. pylori* 26,695 cells were then added to cultures at an MOI ratio of 100 and grown at 37°C for 12 h. Eudesmin (10, 20, 40, 80 and 250 μM) was added to the cells, while only 0.1% DMSO was added to the control group. Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then washed twice in PBS and quenched with PBS (pH 7.4) containing 0.02% Triton X-100 for 30 min, prior to blocking for 30 min at room temperature in PBS with 5% non-fat dry milk. Coverslips were incubated with anti-microtubule-associated protein 1A/1B-light chain 3, isoform B (LC-3B) antibody (cat. no. NB600-1384, dilution 1:200; Novus Biologicals, LLC, Littleton, CO, USA) at room temperature for 1 h. Cells were then washed in PBS for 5 min three times and incubated with secondary anti-rabbit fluorescein isothiocyanate-labeled antibody (cat. no. NB730-F, dilution 1:10,000; Novus Biologicals, LLC) at room temperature in PBS for 1 h. Then, coverslips were washed twice in PBS and incubated with LysoTracker Red DND-99 (cat. no. L7528; Thermo Fisher Scientific, Inc.) at a dilution of 1:1,000 in PBS for 1 h, followed by incubation with 300 nM 4',6-diamidino-2-phenylindole (cat. no. D1306; Thermo Fisher Scientific, Inc.) for 5 min at room temperature. Fluorescent signatures were then visualized using a confocal spectral

microscope (Leica SP2; Leica Microsystems GmbH, Wetzlar, Germany).

Preparation of cell extracts and western blot analysis. AGS cells were seeded onto 6-well plates at a density of 5×10^5 cells/well for 18 h. The cells co-cultured with PBS-resuspended *H. pylori* at MOI of 100 were treated with varying concentrations of eudesmin (10, 20, 40, 80 and 250 μM) or 0.1% DMSO alone (control group) for 6 h in antibiotic-free RPMI 1640 supplemented with 10% FBS. Infected cells were then lysed with ice-cold lysis buffer (0.5 M Tris-HCl, pH 7.4, 10% SDS and 0.5 M dithiothreitol). Protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 20 μg protein samples were loaded on each lane and separated on 12% SDS-PAGE using the Hoefer miniVE system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Proteins were transferred to a Hybond-P PVDF membrane (GE Healthcare Bio-sciences) according to the manufacturer's instructions. Following the transfer, the membrane was washed with PBS and blocked for 1 h at 37°C with 5% non-fat dry milk and 0.1% Tween-20 in PBS (PBST). The primary antibodies [mouse anti- β -actin (cat. no. MAB1501; EMD Millipore, Billerica, MA, USA), rabbit anti-caspase-8 (cat. no. 25901; Abcam, Cambridge, UK), rabbit anti-BH3 interacting domain death agonist (Bid; cat. no. 2002; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-Bcl-2-associated X protein (Bax; cat. no. 2772; Cell Signaling Technology, Inc.), rabbit anti-cytochrome *c* (cat. no. SC-7159), and rabbit anti-caspase-9 (cat. no. SC-7885) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or rabbit anti-caspase-3 (cat. no. AB1899; EMD Millipore)] were added at a dilution of 1:1,000 in PBST. Blots were washed for 5 min three times in PBST and then incubated with the peroxidase-conjugated secondary antibodies [horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (cat. no. SC-2005; Santa Cruz Biotechnology, Inc.) or goat anti rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.)] at a dilution of 1:10,000 in PBS. Following removal of the secondary antibody, blots were washed with PBST (three times, 5 min each) and then developed using a Pierce ECL-Western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.). Densities of the obtained immunoblots were quantified by Kodak digital science 1D (version 2.03; Kodak, Rochester, NY, USA).

Mouse model of *H. pylori* infection. A total of 60 male C57BL/6 mice, obtained from the National Laboratory Animal Center (Taipei, Taiwan), weighing 20-22 g, were maintained in a pathogen-free environment and used when they reached 4 weeks of age. Mice were randomly divided into 6 groups ($n=10$). Mice were housed in an air-conditioned room ($25 \pm 2^\circ\text{C}$) with a relative humidity of 40-70% and were subjected to a 12-h light/dark cycle. Mice had *ad libitum* access to tap water and a standard laboratory rodent diet. All animal-based experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University (approval no. 101-96-N; Taichung, Taiwan) and performed according to the ethical rules and laws of China Medical University. Mice were fed a basal diet (ProLab RMH 2500, 5P14; LabDiet, St. Louis, MO, USA) for 1 week

prior to use in the study. To establish *H. pylori* infection, all mice, apart from those in the control group, were infected with 1×10^9 CFU *H. pylori* 26695 every other day for a total of 3 doses using stomach tubes. All test samples (5, 10, 20 and 40 μ M eudesmin) were dissolved in water and administered orally every day at a volume of 0.2 ml per mouse for 3 days using a stomach tube. The control group received the basal diet, without infection and administered water instead of treatment. The infection group was infected and received the basal diet but without eudesmin treatment. Stomach and blood samples were collected from all groups the day after the last treatment was administered and subsequently, the mice were humanely sacrificed by CO₂ asphyxiation. Blood was taken directly from the heart via microsyringe to determine the expression of IL-1 β and IgM. The stomach samples were homogenized in 1.0 ml of sterile saline, with the aid of a tissue homogenizer, at 4°C. The homogenates were then subjected to mRNA isolation using the total RNA Miniprep Purification kit (GMBiolab Co., Ltd., Taichung, Taiwan) described later on. After standing for 5 min at room temperature, supernatant of the homogenate were processed for *H. pylori* load.

ELISA evaluation of immune responses of *H. pylori* infected tissue and cells. Detection of interleukin (IL)-8 in the supernatant of *H. pylori* 26695 infected human AGS cells was conducted using a human IL-8 ELISA Ready-SET-Go!® kit (eBioscience, Inc., San Diego, CA, USA). IL-1 β and immunoglobulin M (IgM) in *H. pylori* infected mice blood were measured using a mouse IL-1 β ELISA Ready-SET-Go! kit (eBioscience, Inc.) and goat anti-mouse IgM horseradish peroxidase-conjugated antibody (cat. no. A90-101P; Bethyl Laboratories, Inc., Montgomery, TX, USA), at a dilution of 1:10,000 in PBS, respectively. All kits were performed following the manual instructions. Each sample was analyzed individually. Results were calculated as the mean of triplicate readings and expressed as fold-change compared with the control group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. During cell culture, AGS cells and PBS-suspended *H. pylori* 26695 at an MOI ratio of 100 were co-cultured in antibiotic-free RPMI-1640 medium supplemented with 10% FBS. The 6 concentrations of eudesmin were added to the culture and 0.1% DMSO alone was added to the control group. Following 3 h infection, total mRNA was isolated from the AGS cells following the method previously described for detecting cytotoxin associated gene A (*cagA*) gene (21) and expression of the vacuolating cytotoxin A (*vacA*) gene (designed in this study) was also detected. For mice models of *H. pylori* infection, the total mRNA of stomach tissue sample homogenates was isolated using the total RNA Miniprep Purification kit (GMBiolab Co., Ltd.) and reverse transcription (RT) was performed using the Fast-Run HotStart RT-qPCR (AMV) kit (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan). The kits were used following the manufacturer's instructions. The oligonucleotide primers used for RT corresponded with the murine gene sequences. All oligonucleotide primers used were synthesized by Mission Biotech Co., Ltd. (Taipei, Taiwan). RT-qPCR was performed at the following conditions: 10 min at 95°C; 40 cycles of 15 sec at

Table I. Minimal bactericidal concentrations of eudesmin, amoxicillin, clarithromycin, and metronidazole against various *H. pylori* strains.

Type of antibiotic	Minimal bactericidal concentration of <i>H. pylori</i> , μ M				
	Strain	26695	v633	v1254	v1354
Eudesmin		10	5	2.5	10
Amoxicillin		0.5	16	0.25	2
Clarithromycin		0.061	125	62.5	125
Metronidazole		15.625	500	62.5	250

H. pylori, *Helicobacter pylori*.

95°C; and 1 min at 60°C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 200 nM forward (F) and reverse (R) primers (*vacA* F, 5'-CTGGAGCCGGGAGGAAAG-3' and R, 5'-GGCGCCATCATAAAGAGAAATTT-3'; *cagA* F, 5'-ATAATGCTAATTAGACAACCTTGAGCGA-3' and R, 5'-TTAGAATAATCAACAAACATCACGCCAT-3'; 16S RNA of *H. pylori* F, 5'-GTGTGGGAGAGGTAGGTGGA-3' and R, 5'-TGC GTTAGCTGCATTACTGG-3'). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system (Thermo Fisher Scientific, Inc.) and the fold-changes in expression were derived using the comparative $\Delta\Delta C_q$ method (22). 16S RNA of *H. pylori* served as an internal control for sample loading and mRNA integrity, as previously described (21).

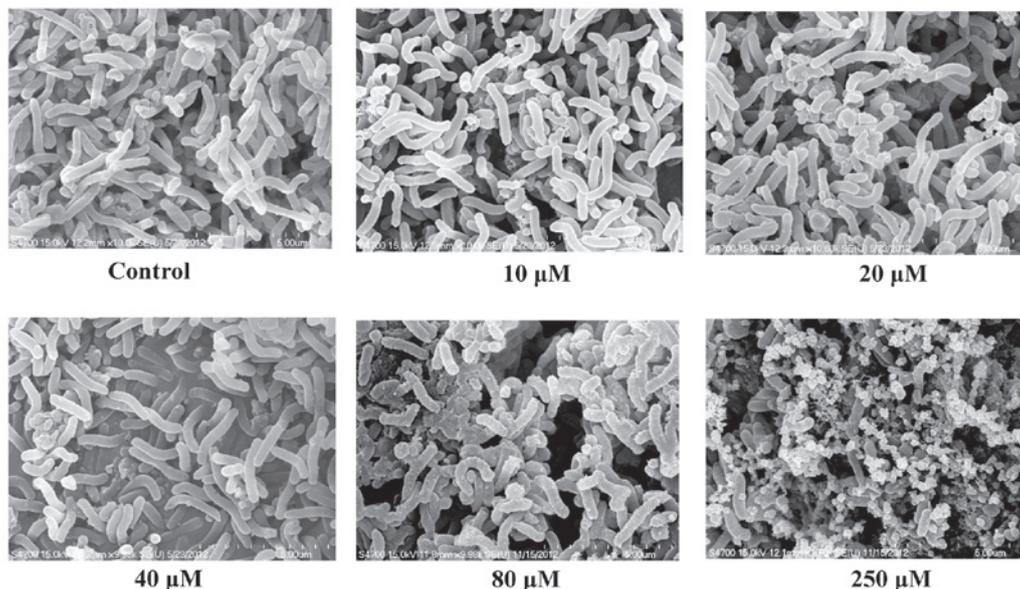
Statistical analysis. The differences between the mean values of groups were evaluated by one-way analysis of variance followed by Duncan's test using SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA). The results were then presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of eudesmin on *H. pylori* in vitro. The anti-*H. pylori* properties of eudesmin were tested against the reference strain 26695 and clinical isolates from *H. pylori*-positive patients who failed following typical antibiotic treatment in previous studies (19,20). All clinical isolate strains exhibited resistance against amoxicillin, clarithromycin and metronidazole (Table I). The MBC of eudesmin against the tested *H. pylori* strains are summarized in Tables I and II. Eudesmin exhibited the best bactericidal activity against antibiotic resistant strain v1254 (MBC, 2.5 μ M) and strain 26695 (MBC, 10 μ M). The bactericidal activity of eudesmin against Gram-negative (*Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium and *Escherichia coli*) and Gram-positive bacteria (*Streptococcus aureus*) were also tested (Table II). The MBCs of eudesmin against all bacteria tested, excluding *H. pylori*, were $>320 \mu$ M. Eudesmin exhibited a strong bacterial activity against the morphology of *H. pylori* in a dose-dependent manner (Fig. 2). Eudesmin at concentration

Table II. Minimal bactericidal concentrations of eudesmin against different gram-negative and gram-positive bacteria.

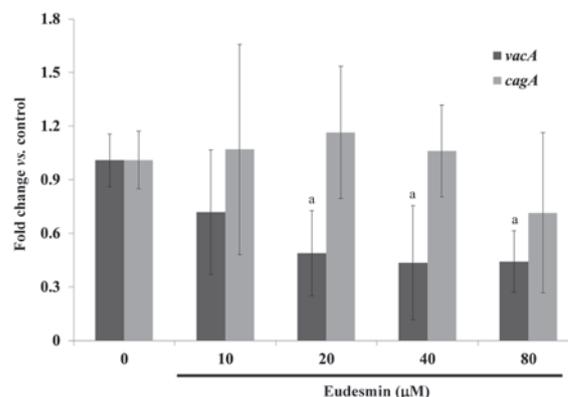
Strain of bacteria	Minimal bactericidal concentration, μM				
	<i>Helicobacter pylori</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella enterica</i> serovar Typhimurium	<i>Escherichia coli</i>	<i>Streptococcus aureus</i>
Eudesmin	10	>320	>320	>320	>320

Figure 2. Effect of eudesmin on the morphology of *H. pylori*. For morphological examination, *H. pylori* 26695 cultures were treated with 0, 10, 20, 40, 80 and 250 μM of eudesmin for 6 h. The morphological examination was determined using a scanning electron microscopy. *H. pylori*, *Helicobacter pylori*.

of 250 μM markedly damaged the architecture of *H. pylori*. Furthermore, eudesmin significantly decreased the expression of *vacA* but not *cagA*, in *H. pylori* at concentrations ≥ 20 μM ($P < 0.05$; Fig. 3). These genes are well-characterized virulence factors of *H. pylori*.

Effect of eudesmin on *H. pylori*-infected human AGS cells in vitro. A cell viability assay was performed in order to study the cytotoxicity of eudesmin. The IC_{50} of eudesmin was 395 μM in AGS cells. These data suggest that eudesmin exerts weak cytotoxic activity. The results of the present study demonstrated that eudesmin reversed *H. pylori*-induced AGS cell morphological changes, particularly at 80 μM (Fig. 4). Eudesmin significantly decreased *vacA* and *cagA* gene expression of *H. pylori*-infected AGS cells compared with those of infected mice that did not receive treatment ($P < 0.05$; Fig. 5A). In addition, at concentrations ≥ 10 μM , eudesmin significantly decreased the ability of *H. pylori* to associate with AGS cells ($P < 0.05$; Fig. 5B). Infection with *H. pylori* can lead to an inflammatory response, causing increased IL-8 expression. It was observed that eudesmin significantly reduced IL-8 expression, and the inflammatory reaction, at concentrations ≥ 20 μM ($P < 0.05$; Fig. 6).

Effect of eudesmin on autophagy and apoptosis in *H. pylori*-infected AGS cells in vitro. The present study

Figure 3. Effect of eudesmin on *H. pylori vacA* and *cagA* mRNA expression. *H. pylori* 26695 cultures were treated with 0, 10, 20, 40 and 80 μM of eudesmin for 6 h. The mRNA levels of *vacA* and *cagA* were determined by reverse transcription-quantitative polymerase chain reaction. Data comparing the eudesmin-treated cells and untreated cells are presented as the mean \pm SD in triplicate. * $P < 0.05$ for *vacA* gene expression vs. non-treatment group. *H. pylori*, *Helicobacter pylori*.

investigated the effect of eudesmin on programmed cell death, through autophagy or apoptosis, in *H. pylori*-infected AGS cells. Eudesmin treatment between 20 and 80 μM notably decreased autophagy-associated LC-3B protein levels (Fig. 7). Treatment with 20-80 μM eudesmin inhibited

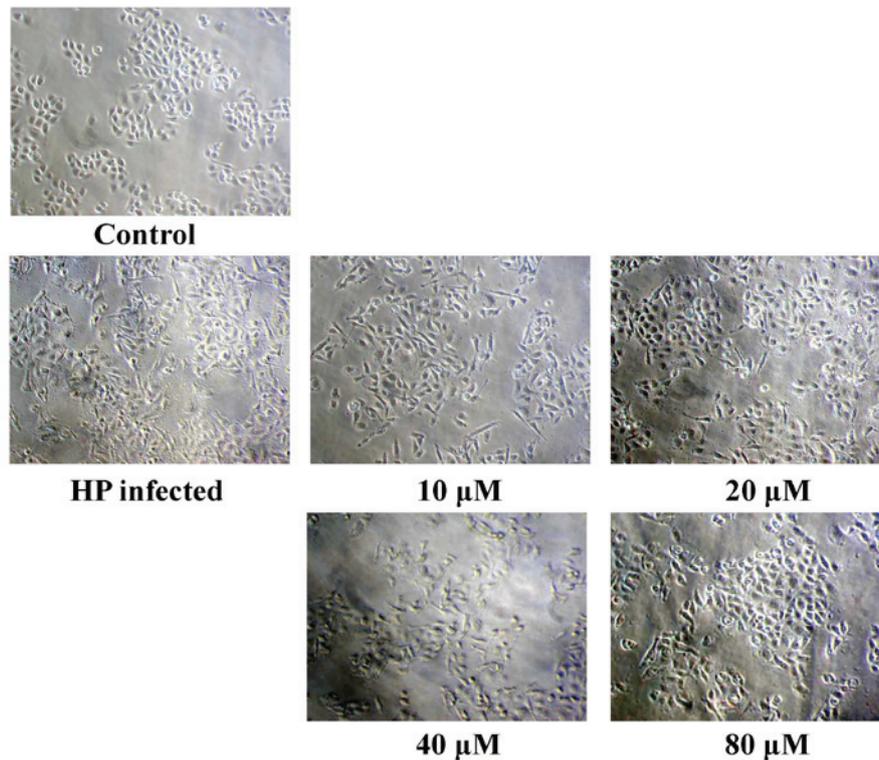


Figure 4. Effect of eudesmin on the morphology in *H. pylori*-infected AGS cells. For morphological examination, *H. pylori* 26695-infected AGS cells were treated with 0, 10, 20, 40, 80 and 250 μM of eudesmin for 6 h. HP, *Helicobacter pylori*.

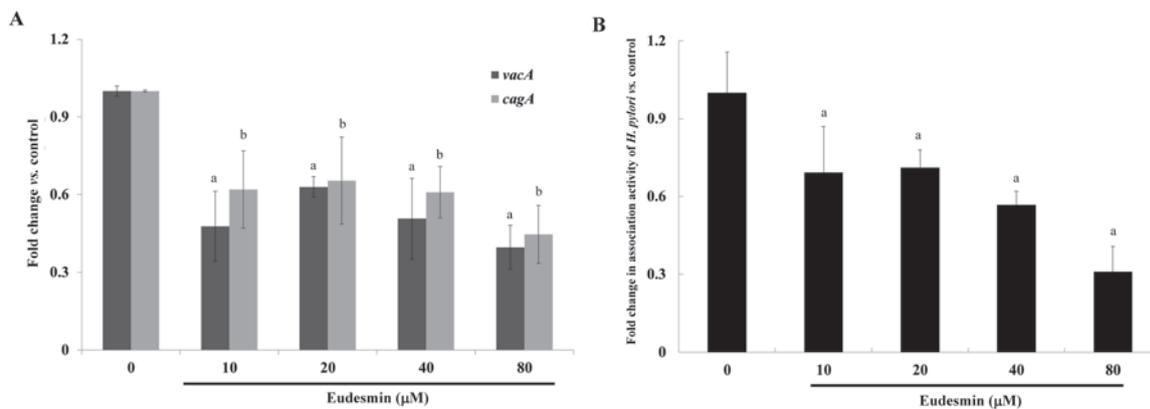


Figure 5. Effect of eudesmin on *H. pylori vacA* and *cagA* expression in and bacterial association to AGS cells. (A) Effect of eudesmin on *vacA* and *cagA* mRNA expression in *H. pylori* 26695-infected AGS cells. *H. pylori*-infected AGS cells were treated with 0, 10, 20, 40 and 80 μM eudesmin for 6 h. Total mRNA levels of *vacA* and *cagA* were determined by reverse transcription-quantitative polymerase chain reaction. ^a $P < 0.05$ for *vacA* gene expression vs. non-treatment group. ^b $P < 0.05$ for *cagA* gene expression vs. non-treatment group. (B) Effect of eudesmin on *H. pylori* 26695 association to AGS cells. *H. pylori*-infected AGS cells were treated with 0, 10, 20, 40 and 80 μM of eudesmin for 6 h. The association ability of *H. pylori* was determined by bacterial cell counting. Data comparing the eudesmin-treated samples and untreated samples are presented as the mean \pm standard deviation in triplicate. ^a $P < 0.05$ vs. untreated group. *H. pylori*, *Helicobacter pylori*.

the expression of apoptosis-associated caspase-8, Bid, Bax, cytochrome *c*, caspase-9 and -3 protein (Fig. 8). These results suggest that eudesmin inhibits autophagy and apoptosis in *H. pylori*-infected AGS cells.

Effect of eudesmin on a mice model of H. pylori infection. To study the effects of eudesmin *in vivo*, eudesmin (5, 10, 20 and 40 μM) was used to treat *H. pylori*-infected mice for 3 days. Results from the present study identified that the lowest dose (5 μM) of eudesmin was sufficient to significantly decrease

H. pylori-load in the stomach tissue of infected mice ($P < 0.05$; Fig. 9). In addition, serum levels of IL-1 β (Fig. 10A) and IgM (Fig. 10B) in *H. pylori*-infected mice were significantly suppressed at concentrations of 20 and 40 μM eudesmin, respectively ($P < 0.05$). These data suggest that eudesmin reduces inflammatory and immune responses to *H. pylori* infection. To summarize the aforementioned results from the *in vivo* and *in vitro* studies (Fig. 11), eudesmin may reduce the virulence of *H. pylori* and also suppress *H. pylori* induced inflammation, autophagy and apoptosis.

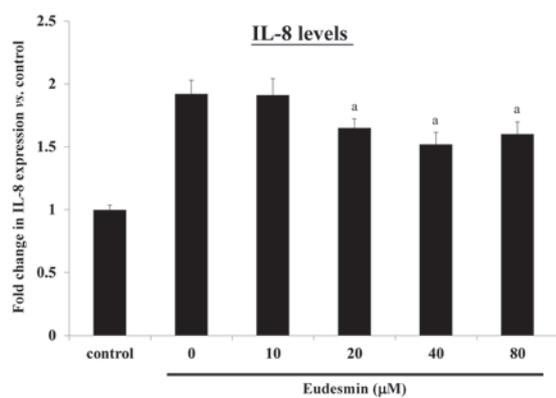


Figure 6. Effect of eudesmin on IL-8 production of *H. pylori*-infected AGS cells. *H. pylori* 26695-infected AGS cells were treated with 0, 10, 20, 40 and 80 µM of eudesmin for 6 h. IL-8 production by the AGS cells was determined by ELISA. Data comparing the eudesmin-treated cells and untreated cells are presented as the mean ± standard deviation in triplicate. ^aP<0.05 vs. untreated group. IL, interleukin; *H. pylori*, *Helicobacter pylori*.

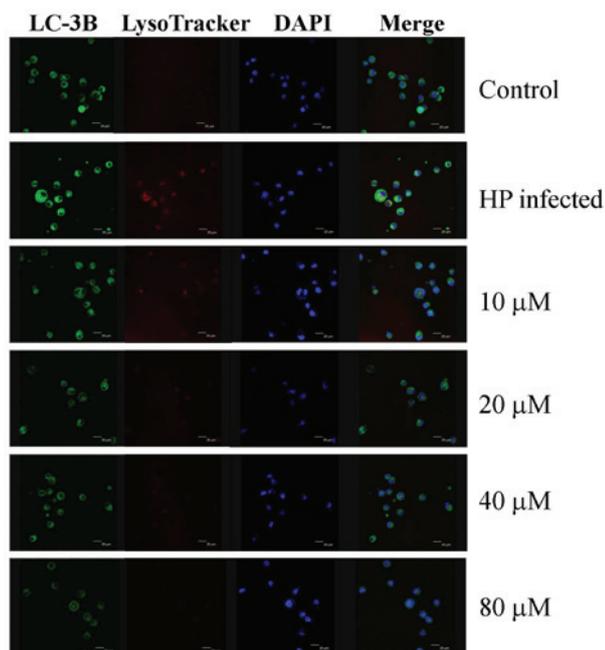


Figure 7. Effect of eudesmin on LC-3B expression in *H. pylori*-infected AGS cells. *H. pylori* 26695-infected AGS cells were treated with 0, 10, 20, 40 and 80 µM of eudesmin for 12 h. Cells were fixed, then stained with LC3-B antibody, and LysoTracker and DAPI dyes. LC-3B protein expression was detected by immunostaining and visualized under a confocal microscope. LC-3B, microtubule-associated protein 1A/1B-light chain 3, isoform B; DAPI, 4',6-diamidino-2-phenylindole; HP, *Helicobacter pylori*.

Discussion

It has previously been demonstrated that eudesmin can be isolated from a number of different plants, including *Apiaceae*, *Rutaceae*, *Ochnaceae* and *Magnoliaceae* (17,18). The present study, to the best of our knowledge, is the first to describe the isolation of eudesmin from *Fatsia polycarpa* Hayata. Numerous previous studies have identified the biological functions of eudesmin, including that its cytotoxic, anti-bacterial, anti-fungal and inhibitory effects on TNF-α

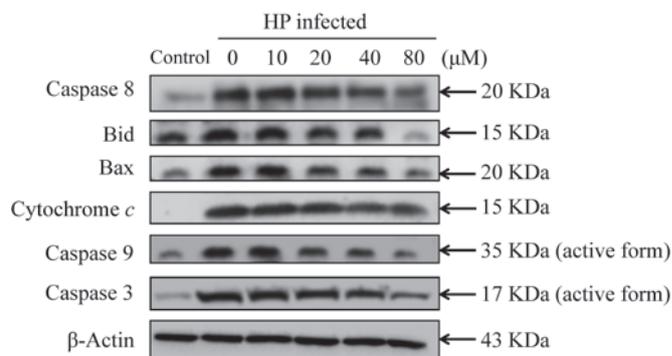


Figure 8. Effect of eudesmin on apoptosis-associated protein levels in *H. pylori*-infected AGS cells. *H. pylori* 26695 infected AGS cells were treated with 0, 10, 20, 40 and 80 µM of eudesmin for 6 h, then subjected to western blotting. Western blotting of caspase-8, Bid, Bax, cytochrome *c*, caspase-9, and -3 protein levels was performed. The β-actin was used as an internal control for equivalent protein loading. HP, *Helicobacter pylori*.

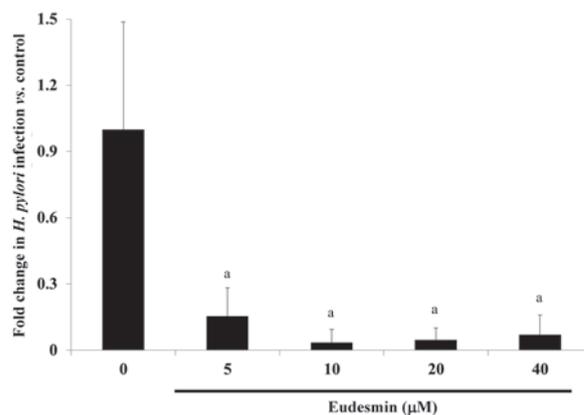


Figure 9. Effect of eudesmin on *H. pylori* load from infected gastric tissues. *H. pylori* 26695-infected C57BL/6 mice were treated with 0, 5, 10, 20 and 40 µM eudesmin for 3 days. The mRNA level of *H. pylori* 16r RNA was determined by reverse transcription-quantitative polymerase chain reaction. Data comparing the eudesmin-treated and untreated control mice are presented as the mean ± standard deviation in triplicate. ^aP<0.05 vs. untreated group. *H. pylori*, *Helicobacter pylori*.

production (13-16). However, the effects of eudesmin on *H. pylori* infection have not been tested previously. The present study, to the best of our knowledge, is the first to investigate the effects of eudesmin on *H. pylori*-infected AGS cells *in vitro* and to study the possible mechanisms involved in eudesmin's anti-bacterial activity.

In the present study, the lowest MBC of eudesmin was 2.5 µM against the antibiotic resistant strain v1254 of *H. pylori*. However, the IC₅₀ of eudesmin was 395 mM in AGS cells and the MBC of eudesmin against other common Gram-negative and Gram-positive bacteria was >320 µM. These results suggest that eudesmin is specifically bactericidal against *H. pylori* but has low cytotoxicity. At a concentration of 250 µM, eudesmin could abolish the morphological structure of *H. pylori* under visualized under a scanning electron microscope. The mechanisms underlying the destruction of the morphology and bactericidal activity against *H. pylori* by eudesmin should be further investigated. Following the treatment of *H. pylori* 26695 with eudesmin, expression of *vacA*, but not *cagA*, was

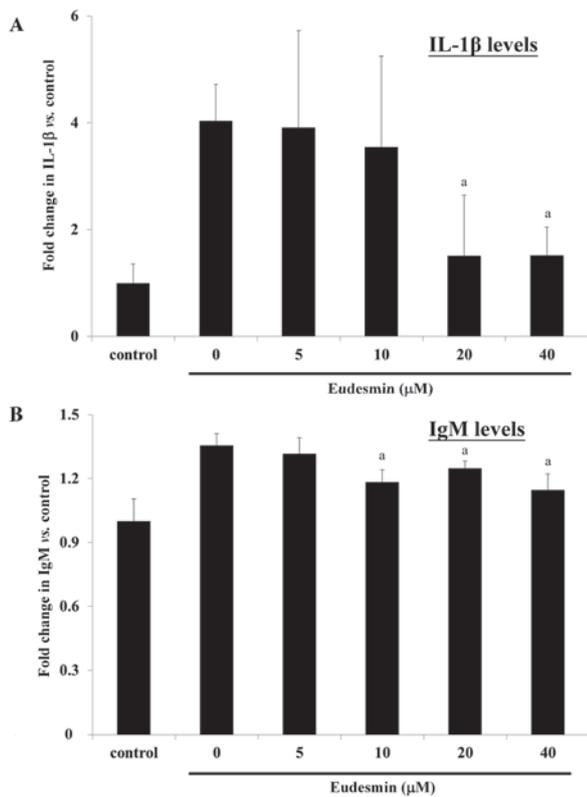


Figure 10. Effect of eudesmin on (A) IL-1β expression and (B) IgM expression in *H. pylori*-infected C57BL/6 mice *in vivo*. *H. pylori* 26695-infected C57BL/6 mice were treated with 0, 5, 10, 20 and 40 μM of eudesmin for 3 days. The level of IL-1β and IgM expression was determined by ELISA. Data comparing the eudesmin-treated and non-treated mice are presented as the mean ± standard deviation in triplicate. *P<0.05 vs. non-treatment group. IL, interleukin; IgM, immunoglobulin M.

suppressed. The protease VacA is a well-known virulence factor of *H. pylori*, which causes infected cells to undergo apoptosis (23,24). In addition, VacA serves a role in autophagy in infected cells (24). Interestingly, in *H. pylori*-infected human AGS cells, eudesmin interfered with *vacA* and *cagA* gene expression. CagA protein is delivered into the host cell by a type IV secretion system of *H. pylori*, where it induces the expression of proinflammatory cytokines (25). Therefore, data from the present study indicates that eudesmin can cause damage to *H. pylori* bacteria and interfere with its expression of certain virulence factors while infection occurs.

It has previously been reported that *H. pylori* can induce autophagy in gastric epithelial cells and professional phagocytes (10,11). Deen *et al* (26) reported that *H. pylori* infection can induce canonical autophagy in macrophages. In addition, *H. pylori* has been reported to induce gastric epithelial cell apoptosis through the activation of the cell-surface death receptor pathway and the mitochondrial pathway (9,10). These pathways activate caspase-3 to initiate apoptosis. Furthermore, it has been observed that the VacA protein of *H. pylori* is involved in inducing apoptosis and autophagy in gastric epithelial cells during infection (12). Since eudesmin interferes with the expression of VacA, the positive outcome of eudesmin treatment in *H. pylori* infection models may be due to the fact that the virulence of *H. pylori* is attenuated. In addition, the present study identified that eudesmin

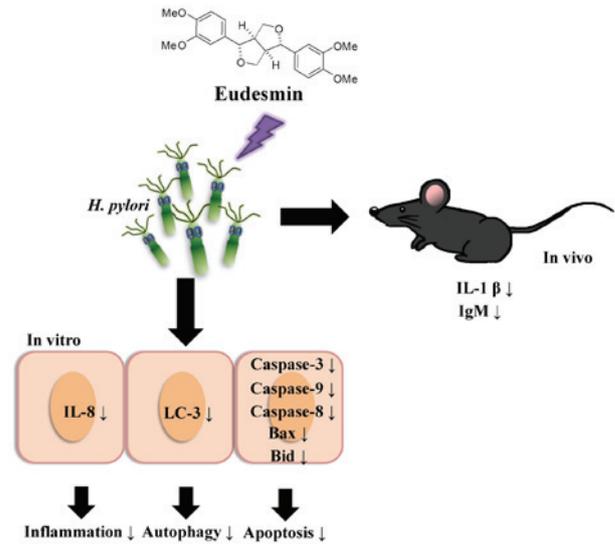


Figure 11. Proposed mechanism by which eudesmin may suppress *H. pylori*-triggered inflammation, autophagy and apoptosis. *H. pylori*, *Helicobacter pylori*.

decreased the expression of proteins involved in apoptosis and autophagy of *H. pylori*-infected AGS cells, such as LC-3B, caspase-3, caspase-9, caspase-8, Bax, and Bid, suggesting that it suppresses *H. pylori*-induced apoptosis and autophagy. Increased apoptosis is associated with the development of gastric carcinoma. Thus, eudesmin may prevent the development gastric carcinoma in *H. pylori*-infected individuals.

H. pylori infection may result in gastritis (1,2). The pathogenesis of gastritis involves the host cell's inflammatory response. Inflammation is the primary host response against microbial infections (4,5). Multiple pathways are involved that mediate the activation of caspases, which subsequently induce the secretion of pro-inflammatory cytokines, such as IL-1β and IL-8 (8). IL-1β is an important pro-inflammatory cytokine that is a powerful inhibitor of gastric acid secretion (27). It has been demonstrated that the expression of IL-1β in gastric mucosa is upregulated following *H. pylori* infection (8) and IL-1β may serve a central role in the initiation of the inflammatory response to infection. Previous studies have reported that *H. pylori* induces the expression of caspase-1 and IL-1β in macrophages and dendritic cells (4,8). The present study determined the effects of eudesmin on *H. pylori*-mediated inflammation in the AGS human gastric adenocarcinoma epithelial cell line. Eudesmin treatment efficiently reduced IL-8 expression by AGS cells in response to *H. pylori* infection. It was also observed that eudesmin decreased IL-1β expression in a mouse model of *H. pylori* infection.

The results of the present study indicate a proposed mechanism by which eudesmin suppresses *H. pylori*-triggered inflammation, autophagy and apoptosis. The results of the present study suggest that *H. pylori* infection induces inflammation in AGS cells, which results in an upregulation of IL-8 and IL-1β *in vitro* and *in vivo*. In conclusion, the present study demonstrates that the administration of eudesmin efficiently eradicates *H. pylori* and attenuates *H. pylori*-induced epithelial cell death through autophagy and apoptosis. The high

efficacy of the eudesmin treatment observed makes eudesmin a promising novel non-antibiotic therapy of *H. pylori* infection.

Acknowledgements

The present study was supported by the Department of Chinese Medicine and Pharmacy, Ministry of Health and Welfare (CCMP99-RD-208), Ministry of Science and Technology, Taiwan NSC-101-2621-B-039-003, China Medical University (CMU103-S-05) and Yen Tjing Ling Medical Foundation (CI-103-21).

References

- de Bernard M and Josenhans C: Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 19 (Suppl 1): S11-S18, 2014.
- Kountouras J, Zavos C, Gavalas E and Tzilves D: Challenge in the pathogenesis of autoimmune pancreatitis: Potential role of *Helicobacter pylori* infection via molecular mimicry. *Gastroenterology* 133: 368-369, 2007.
- Ding SZ, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, Dirden-Kramer B, Boldogh I, Ernst PB and Crowe SE: *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infect Immun* 75: 4030-4039, 2007.
- Clevers HC and Bevins CL: Paneth cells: Maestros of the small intestinal crypts. *Annu Rev Physiol* 75: 289-311, 2013.
- Peek RM Jr, Fiske C and Wilson KT: Role of innate immunity in *Helicobacter pylori*-induced gastric malignancy. *Physiol Rev* 90: 831-858, 2010.
- Federico A, Gravina AG, Miranda A, Loguercio C and Romano M: Eradication of *Helicobacter pylori* infection: Which regimen first? *World J Gastroenterol* 20: 665-672, 2014.
- Nam SY, Park BJ, Ryu KH and Nam JH: Effect of *Helicobacter pylori* infection and its eradication on the fate of gastric polyps. *Eur J Gastroenterol Hepatol* 28: 449-454, 2016.
- Yang JC, Yang HC, Shun CT, Wang TH, Chien CT and Kao JY: Catechins and sialic acid attenuate *Helicobacter pylori*-triggered epithelial caspase-1 activity and eradicate *Helicobacter pylori* infection. *Evid Based Complement Alternat Med* 2013: 248585, 2013.
- Castano-Rodriguez N, Kaakoush NO, Goh KL, Fock KM and Mitchell HM: Autophagy in *Helicobacter pylori* infection and related gastric cancer. *Helicobacter* 20: 353-369, 2015.
- Labbé K and Saleh M: Cell death in the host response to infection. *Cell Death Differ* 15: 1339-1349, 2008.
- Liu WH, Liu TC and Mong MC: Antibacterial effects and action modes of asiatic acid. *Biomedicine (Taipei)* 5: 16, 2015.
- Raju D, Hussey S, Ang M, Terebiznik MR, Sibony M, Galindo-Mata E, Gupta V, Blanke SR, Delgado A, Romero-Gallo J, *et al*: Vacuolating cytotoxin and variants in Atg16L1 that disrupt autophagy promote *Helicobacter pylori* infection in humans. *Gastroenterology* 142: 1160-1171, 2012.
- Liu H, Song Z, Liao DG, Zhang TY, Liu F, Zhuang K, Luo K, Yang L, He J and Lei JP: Anticonvulsant and sedative effects of eudesmin isolated from *Acorus tatarinowii* on mice and rats. *Phytother Res* 29: 996-1003, 2015.
- Cho JY, Yoo ES, Baik KU and Park MH: Eudesmin inhibits tumor necrosis factor-alpha production and T cell proliferation. *Arch Pharm Res* 22: 348-353, 1999.
- Raimundo JM, Trindade AP, Veloze LS, Kaplan MA, Sudo RT and Zapata-Sudo G: The lignan eudesmin extracted from *Piper truncatum* induced vascular relaxation via activation of endothelial histamine H1 receptors. *Eur J Pharmacol* 606: 150-154, 2009.
- Yang YJ, Park JI, Lee HJ, Seo SM, Lee OK, Choi DH, Paik KH and Lee MK: Effects of (+)-eudesmin from the stem bark of magnolia kobus DC. var. borealis Sarg. on neurite outgrowth in PC12 cells. *Arch Pharm Res* 29: 1114-1118, 2006.
- Wang CM, Chen HT, Li TC, Weng JH, Jhan YL, Lin SX and Chou CH: The role of pentacyclic triterpenoids in the allelopathic effects of *Alstonia scholaris*. *J Chem Ecol* 40: 90-98, 2014.
- Batista AND, Batista JM, Lopez SN, Furlan M, Cavalheiro AJ, Silva DHS, Bolzani VDS, Nunomura M and Yoshida M: Aromatic compounds from three Brazilian Lauraceae species. *Quim Nova* 33: 321-323, 2010.
- Poon SK, Chang CS, Su J, Lai CH, Yang CC, Chen GH and Wang WC: Primary resistance to antibiotics and its clinical impact on the efficacy of *Helicobacter pylori* lansoprazole-based triple therapies. *Aliment Pharmacol Ther* 16: 291-296, 2002.
- Lai CH, Kuo CH, Chen PY, Poon SK, Chang CS and Wang WC: Association of antibiotic resistance and higher internalization activity in resistant *Helicobacter pylori* isolates. *J Antimicrob Chemother* 57: 466-471, 2006.
- Li L, Kelly LK, Ayub K, Graham DY and Go MF: Genotypes of *Helicobacter pylori* obtained from gastric ulcer patients taking or not taking NSAIDs. *Am J Gastroenterol* 94: 1502-1507, 1999.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- da Costa DM, Pereira Edos S and Rabenhorst SH: What exists beyond cagA and vacA? *Helicobacter pylori* genes in gastric diseases. *World J Gastroenterol* 21: 10563-10572, 2015.
- Torres J and Backert S: Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 13 (Suppl 1): S13-S17, 2008.
- Alzahrani S, Lina TT, Gonzalez J, Pinchuk IV, Beswick EJ and Reyes VE: Effect of *Helicobacter pylori* on gastric epithelial cells. *World J Gastroenterol* 20: 12767-12780, 2014.
- Deen NS, Huang SJ, Gong L, Kwok T and Devenish RJ: The impact of autophagic processes on the intracellular fate of *Helicobacter pylori*: More tricks from an enigmatic pathogen? *Autophagy* 9: 639-652, 2013.
- El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, *et al*: Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404: 398-402, 2000.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.